

CELLULAR IMMUNE RESPONSE TO OUTER MEMBRANE PROTEINS ISOLATED FROM *ACINETOBACTER BAUMANNII*

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ABSTRACT

A total of 458 samples were collected during 2012, distributed in urine, blood, sputum, swabs from wound infection and swabs from burn. The results revealed that 11 (2.4%) isolates obtained belonged to *Acinetobacter baumannii*, which were collected from various clinical samples distributed in blood 1 (0.93%), urine 2 (1.44%), wound infection 2 (3.12%) and burn 6 (6.25%). These isolates were identified by using microscopic examination, biochemical tests, and vitek 2 system. The SDS-PAGE method was used to analysis outer membrane proteins profile revealed that the molecular weight was around 44 KD. Immunization was carried out for three rabbits groups after preparation outer membrane proteins (OMPs) and killed antigen used for first two groups while a third group represented a control group which was injected with normal saline. At the end of the immunization period then itrobluetetrazolium, E-rosette, were used to investigate the cellular immune response which revealed a significant difference in cellular immune response for rabbits immunized with OMPs and killed antigens in compare with control group.

KEYWORDS: *A. baumannii*, *Oryctylagus conniculus*, Biochemical Tests

INTRODUCTION

Acinetobacter baumannii represents the most clinically important and frequently detected *Acinetobacter* species(1) *Acinetobacter baumannii* is a glucose non-fermentative Gram-negative coccobacillus bacterium (2), has become an increasingly prevalent cause of nosocomial infections especially in immune compromised and in Intensive Care Units (ICUs)(3). The outbreak of *A. baumannii* associated with United States military operations in Iraq generated special interest in this organism(4). *A. baumannii* of clinical isolates are commonly resistant to multiple antimicrobial drug classes and have the ability to survive in the environment for prolonged periods of time, which facilitates their persistence in hospitals(5). The Gram-negative bacteria including *A.baumannii* contain a cell envelope composed of two membranes, outer and inner membranes, outer membrane of a Gram-negative bacterium is a protective structure that shields the bacterium from external hazards such as detergents and foreign enzymes. OMPs have been shown to contribute to the virulence of *A. baumannii*. OMPs was implicated to facilitate adhesion, particularly to epithelial cells from the airways (6). OMPs, induces maturation and activation of dendritic cells and directs differentiation of the CD4+ cells toward Th1 polarity (7).

MATERIALS AND METHODS

A total of 458 samples were collected during 2012, the sample were distributed in urine, blood, sputum, swabs from wound infection and swabs from burn. Preliminary identification was performed by gram staining, culturing on MacConkey's agar (Himedia, India) and incubated for overnight at 37°C, non lactose fermenting bacteria or colorless were subcultured and incubated for additional overnights. Suspected bacterial isolates which their cells are Gram negative coccobacillary and negative to oxidase, positive catalase further identified by the traditional biochemical test according to(8) and (9) then additional confirmed by vitek 2.

Isolation of Outer Membrane Proteins of *A. Baumannii*

OMPs of *A. baumannii* were prepared according to the method of (10) with few modifications from 2-3litter of brain heart infusion broth was inoculated by *A. baumannii* incubated for 24- 48 hour at 37 ° C in a shaker incubator, after incubation period bacteria cultured was distributed in sterile plain tube and harvested by centrifugation at 6000 rpm for 15 min, obtained pellet was washed twice by 40 ml of phosphate buffer saline (PBS, pH 7.2), once in 40 ml of Hepes buffer (pH 7.4) by using polyethylene tubes during centrifuge at 10000 rpm for 10 min after mixing by vortex. The cells were resuspended in (20) ml Hepes buffer by vortex and disrupted by sonication for 30 min at 10 watt an interval of 30 second with ice.

Unbroken cells and cellular debris were removed by centrifugation at 4000rpm for (15) min at 4°C, resultant supernatant was placed in polyethylene tubes then further centrifuged at 10000rpm for 1 hour at 4°C. The supernatant was discarded and the pellet suspended in 20 ml of 2% triton X-100 and incubated at room temperature for 30 min to solubilize the inner membrane. The suspension was then centrifuged at 10000rpm for 1 hour at 4°C. The supernatant was discarded and the pellet suspended in 2-5 of normal saline then used or stored at -20°C until use.

SDS-Page Analysis of Outer Membrane Proteins (OMPs)

SDS-page were prepared according to the method of (11). with few modification, OMPs sample were diluted with sample buffer in ratio of 4:1 and heated at 95 °C for 5 min, 30 µl was loaded in each lane of the gel. The gel was run at 150v for 6 hour, then stained with coomassie brilliant blue R-250 staining solution in tray for overnight. Distained in tray with distaining solution.

Preparation Heat Killed Whole Cell Bacterial Antigen

Heat killed whole cell bacterial antigen prepared according to the (12). Tube of 10 ml nutrient broth media was inoculated with 2-4 of a young bacterial isolates incubated at 37°C for 24-48 hours. The culture was centrifuged at 4000rpm for 15min. The supernatant was discarded and the sediment was washed three times in 5ml of phosphate buffer saline (PBS) by using centrifuge at (3500) rpm for (5) min after mixing by vortex. The sediment was resuspended in 5ml of PBS by vortex then suspension was put in water bath at 70°C for 1hour then the bacterial suspension cultured on nutrient agar media for 24 hour at 37°C to make sure all bacteria was killed. The killed bacterial suspension was centrifuged at 4000rpm for (5)min then the sediment was suspension in 3 ml of normal saline and used or stored in -20°C until use.

Laboratory Animals

Nine of local rabbits (*Oryctylagus conniculus*) were left for two weeks to adapted conditions in animal house. Divided into three groups, each group composed of three animals. First group was injected with killed antigen and second group was injected with outer membrane proteins antigen while the third group was injected with normal saline only considered as control group.

Blood Samples

In fifth week after immuazation 3 ml blood from the heart had been withdrawn in a way heart puncture by using sterile syringes. The blood samples were placed in EDTA tube as anticoagulant which processed for immunological study.

Cellular Immune Function Tests

Nitrobluetetrazolium (NBT) Dye Reduction Test

Rabbit blood 0.1ml was mixed with 0.1ml of NBT solution in a well of micro titer plate. The mixture was mixed

gently and covered to ensure humidity, and incubated at 37°C for (15- 25)min. Smear was then made and stained with wright stain and left for 5min. then rinsed with D.W and allowed to dry.

The slide was examined under oil immersion. This test is used to detect phagocytic activity of phagocytic cells as following equation (13).

$$\text{Phagocytic cells index} = \frac{\text{NO.of phagocytic cells reducing to dye}}{\text{Total of phagocytic cells}} \times 100\%$$

E-Rosette Forming Test

The E-rosette test was done according to the method of (14). **a-**Separation of peripheral blood lymphocytes, two ml of heparinized blood was gently mixed with 4ml of hepes-BSS, the diluted blood was carefully added to the 2ml Hisep LSM medium (Hi Media), alongside the inner edge of the tube, and centrifuged at 1500-2000 rpm for 30 min. Four layers appeared including diluted plasma, lymphocytes, lymphoprep (Hisep LSM medium) and the final layer which consists of red blood cells granulocytes and platelets. **b-** Rosettes were prepared as described earlier (15). **c-**Microscopic examination of cell suspension. A cover slip preparation was made and 200 lymphocytes were counted to calculate the percentage of rosettes forming lymphocytes. Lymphocytes covered with three or more sheep red blood cells were taken as rosettes.

Statistical Analysis

Data were processed and analyzed with independent T-test using statistical package of social science19 and the results were expressed as (Mean \pm S.D). P-values below 0.05 were considered to be statistically significant (16).

RESULTS

Distribution of *Acinetobacter baumannii* According to Infection

The results revealed that from (458) clinical sample, a total of (11) (2.4%) isolates obtained belonged to *Acinetobacter baumannii*. However, the distribution of the *Acinetobacter baumannii* isolates and their sites of isolation are listed in table 2.

The result in this table showed that (11) at (2.4%) clinical isolates of *A.baumannii* were collected from various clinical samples distributed in blood 1 (0.93%), urine2 (1.44%), wound infection 2 (3.12%), burn6 (6.25%).

Table 1: Distribution of *A.baumannii* According to Infection

Type of Infection (Sample)	No. of Sample	No. of Positive Isolate	%
Urinary Tract Infection (Urine)	138	2	1.44
Septicemia (Blood)	107	1	0.93
Respiratory Tract Infection (Sputume)	53	0	0
Wound Infection (Swab)	64	2	3.12
Burn Infection (Swab)	96	6	6.25
Total	458	11	2.40

Characterization of Outer Membrane Protein of *A.baumannii* by Using SDS-PAGE

The protein profiles were studied by 10% SDS-PAGE with molecular weight standards used as a size marker. The protein concentration was estimated after reconstitution in buffer and approximately 30 μ g protein was loaded into each well. After electrophoresis, the gels were stained with Coomassie Blue R-250. In SDS-PAGE analysis of the OMP of *A.baumannii* revealed that the molecular weight (MW) is 44KD which estimated by comparison with standard MW markers. (figure 1)

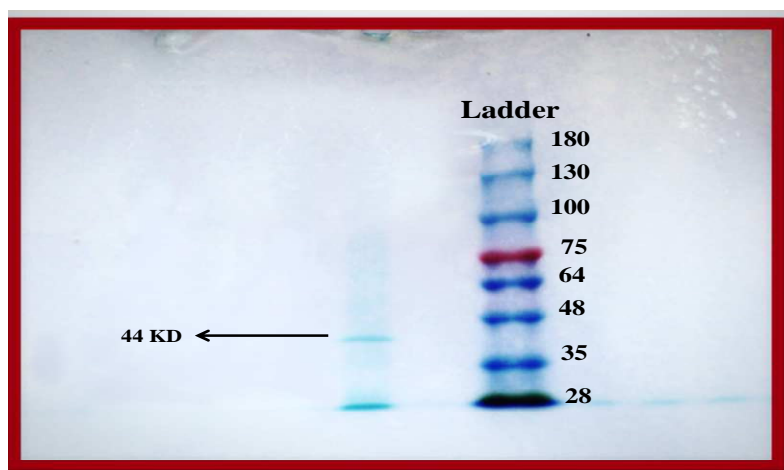


Figure 1: Proteins Profile. Proteins from SDS-Page (10% W/V) of Whole Cell Lysate Preparation from *A. baumannii* at 150v for 6 Hours. Lane (L): Protein Ladder 10-180 KD, Arrow: 44-KD Band

Immunological Study

Assessment of Phagocytic Activity

The results showed increase phagocytic activity of rabbits immunized with OMPs Ag reached (69 %) while the mean value of the phagocytic activity was (58%) for killed bacteria. Statistical analysis revealed significant difference ($P < 0.05$) in phagocytic activity between rabbits immunized with OMPs and killed antigen. While the control group showed a significant decrease ($P < 0.05$) in phagocytic activity at mean value (34.3%) in compared with OMPs and killed bacteria antigen as see in table 2.

Table 2: Phagocytic Activity in Rabbits Immunized with *A.baumannii* Antigens

Antigen	No. Animal	Phagocytic Activity (%) Mean \pm S.D	P-Value [†]
OMPs	3	69 \pm 1.00	a0.004*
Killed bacteria	3	58 \pm 3	b0.003*
Normal saline	3	34.3 \pm 5.85	c0.001*

* $P < 0.05$ significance. [†] Values of P are ^a: for OMP compared with killed antigen, ^b: for killed bacteria compared with control ^c: for control compared with OMPs, S.D=Standard deviation

Erythrocyte-Rosette Formation Test (E-Rosette Test)

This formation occurs due to an immunological reaction between an epitope on the central cells surface and a receptor or antibody on a red blood cell. There are significant increases in mean values of E- rosette for OMP and killed bacteria antigen groups in comparison with control group ($P < 0.05$), and also there is significant difference for OMPs antigen in compare with killed bacteria antigen table 3.

Table 3: The Percentage of E- Rosette Forming in Rabbits Immunized with *A.baumannii* Antigens

Antigen	Systemic E-Rosette (%) Mean \pm S.D	P-Value [†]
OMPs	52.00 \pm 2.00	a.049*
Killed bacteria	47.33 \pm 2.08	b.000*
Normal saline	29.66 \pm 1.52	c.000*

* $P < 0.05$ is significance ^a: for OMP compared with killed bacteria antigen, ^b: for killed bacteria antigen compared with control, ^c: for control compared with OMPs, S.D=Standard deviation

DISCUSSIONS

The intense band in the gel is 44KD figure 1 (17) said that major and significant band of the OMPs of *A. baumannii* on SDS-PAGE is a 40 KD. *A. baumannii* contains other major proteins having molecular masses of 33, 36, and 44 KD accompanied by traces of other proteins, notably at 22, 28, and 30KD, and 50 KD. The total OMPs from the SDS insoluble fraction consisted of proteins with 44 KD and no detectable concentration of proteins above 40 KD was observed this may be due to the digested of peptidoglycan by lysozyme releasing proteins with smaller molecular weight. (18). The enhancement of cellular immune response after OMPs immunization, can be explained by the fact that OMPs are biological active molecules that activate immune cell via toll – like receptors (19). In table (2, 3) results showed an increase in phagocytic activity and mean values of E- rosette forming cells for rabbits immunized with OMPs Ag and killed bacteria in compare with control group at ($P < 0.05$) that comparable with (20,21) who founds that immunization of rabbits with OMP of *Klebsiella pneumoniae*, *Citrobacter freundii* respectively lead to increasing in phagocytic activity and E-rosette formation.

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